

Short Communication

PCR-RFLP of *Ficus deltoidea* Jack (Moraceae)

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ABSTRACT

Previous taxonomic determination of *Ficus deltoidea* Jack was mainly based on narrative morphology. However, the classification and relationship of some *F. deltoidea* varieties were disputable. In this study, four universal primers were used to amplify chloroplast DNA and were digested with eight restriction enzymes to obtain intravarietal variation among *Ficus deltoidea* var. *deltoidea*, *Ficus deltoidea* var. *trengganuensis*, *Ficus deltoidea* var. *intermedia*, *Ficus deltoidea* var. *kunstleri*, *Ficus deltoidea* var. *angustifolia*, and *Ficus deltoidea* var. *bilobata*. Pre-screening of six cpDNA primers revealed that the *rbcL* and *rpoB* amplified fragments contained restriction sites for *Hind*III and *Rsa*I. However, no polymorphism was observed by using this method.

Keywords: *Ficus deltoidea* Jack, chloroplast DNA, PCR-RFLP

ABSTRAK

Penentuan taksonomi bagi *Ficus deltoidea* Jack secara asasnya adalah berdasarkan kepada kaedah morfologi. Walaubagaimanapun, pengelasan dan perhubungan antara beberapa varieti *Ficus deltoidea* telah dipertikaikan. Dalam kajian ini, sebanyak empat pencetus universal telah dipilih dan digunakan untuk mengamplifikasi kawasan DNA kloroplas dan kemudian dicernakan oleh lapan enzim penyekat untuk mendapatkan variasi intravarieti di antara *Ficus deltoidea* var. *deltoidea*, *Ficus deltoidea* var. *trengganuensis*, *Ficus deltoidea* var. *intermedia*, *Ficus deltoidea* var. *kunstleri*, *Ficus deltoidea* var. *angustifolia* dan *Ficus deltoidea* var. *bilobata*. Kawasan cpDNA, telah diamplifikasikan dengan menggunakan enam pencetus cpDNA tetapi hanya pencetus *rbcL* dan *rpoB* mempunyai tapak penyekatan untuk *Hind*III dan *Rsa*I. Namun, tiada polimorfisma yang dapat dicerap dengan menggunakan kaedah ini.

Kata kunci: *Ficus deltoidea* Jack, DNA kloroplas, PCR-RFLP

INTRODUCTION

Ficus deltoidea Jack, locally known as *Mas Cotek* is believed to possess traditional medicinal value (Mat-Salleh and Latiff, 2002). It belongs to the family Moraceae. In recent years, they have been cultivated for ornamental and natural product purposes. The most distinctive features of *F. deltoidea* are the presence of fine golden spots and forked midrib on the leaves, as well as a yellow-olive leathery structure on the dorsal surface (Kochummen, 1978).

Corner (1969) is the pioneer taxonomist in publishing a well-documented work about *Ficus deltoidea*. He classified *F. deltoidea* into 13 varieties based on the morphological variations of leaves and figs. The distribution and ecology of *F. deltoidea* were also recorded in his study. However, the classification is vague as two or more varieties were demonstrated to share similar characteristics, and some of the collections showed intermediate characteristics. For example, leaf morphological variations in var. *intermedia* can range from penninerved lamina to obtuse-spatulate to oblanceolate; as found in var. *motleyana* (Berg, 2003). Leaf shape heterophylly, where leaf shape at the lower plant part was different from the upper and middle plant parts, was observed in *intermedia*, *bilobata*, *trengganuensis* and *angustifolia* varieties but not in *deltoidea* and *kunstleri* varieties. Leaf apex heterophylly was also observed in the six varieties studied (Nashriyah *et al.*, 2010).

Kochummen (1978) stated that only seven varieties can be found in Malaysia, namely *F. deltoidea* var. *kunstleri*, *F. deltoidea* var. *bilobata*, *F. deltoidea* var. *angustifolia*, *F. deltoidea* var. *deltoidea*, *F. deltoidea* var. *trengganuensis*, *F. deltoidea* var. *motleyana* and *F. deltoidea* var. *intermedia*. Most recently, Berg (2003) and Berg and Corner (2005) emerged with a theory that *F. deltoidea* can be divided into subspecies *motleyana* and subspecies *deltoidea*, by excluding var. *motleyana* from subspecies *deltoidea*. Thus, the debates on classification of *F. deltoidea* and *kunstleri* varieties. Leaf apex heterophylly was also observed in the six varieties studied (Nashriyah *et al.*, 2010).

Molecular techniques are suggested as the new approaches to determine variation or relationship in the *F. deltoidea* complex. Polymerase Chain Reaction - Restriction Fragment Length Polymorphism (PCR-RFLP) is one of the molecular techniques used to study molecular systematics. It involves the fragmentation of DNA by restriction enzymes and fragment analysis. The difference in the size of DNA fragments generated is caused by silent mutation that occurs naturally in the organism, and this information is useful to study evolution between species and variety.

MATERIALS AND METHODS

Sample Collection

Ficus deltoidea samples were collected from several locations in Kelantan, Terengganu and Pahang (Table 1). All of the samples were planted and maintained as living collections at the Nursery of Universiti Sultan Zainal Abidin (UniSZA), Gong Badak Campus, Kuala Terengganu, Terengganu, Malaysia. Voucher specimens were retrievable from the UniSZA's Herbarium and some duplicates were deposited at the Herbarium of Universiti Kebangsaan Malaysia (UKMB), Bangi, Selangor, Malaysia. Fresh leaves were kept in plastic bags and stored at -20 °C until further use.

Table 1: *Ficus deltoidea* samples used in this study.

Variety	Sample designation
<i>kunstleri</i>	FD003, FD005, FD 008, FD009, FD010
<i>bilobata</i>	FD013, FD014
<i>angustifolia</i>	FD016, FD017, FD074, FD075
<i>deltoidea</i>	FD006, FD007, D1, D2
<i>trengganuensis</i>	FD004, FD019, FD020, FD021, FD030
<i>intermedia</i>	FD184, FD185, FD186, FD187, FD188

DNA Isolation and Purification

Approximately 1-2 g of *F. deltoidea* leaves were ground to fine powder by using liquid nitrogen (Doyle and Doyle, 1990). Exactly 2% (v/w) CTAB, 1% PVP, 1% PEG and β -mercaptoethanol were added to the powder and immediately placed in a 60 °C incubator for 60 minutes. Supernatant was collected after high speed centrifugation at 12000 rpm. Phenol-chloroform isoamylalcohol was then added to this supernatant, followed with a second centrifugation at 12000 rpm, before adding chloroform isoamylalcohol and 2-propanol. The obtained pellet was then washed with 76%

ethanol:10 mM ammonium acetate, and solubilized with 50 μ L of TE buffer. Exactly 10 μ g/mL RNase was added to the solution and incubated at 37 °C for 30 minutes and further washed with 2.5 M ammonium acetate and cold ethanol. Finally, 50 μ L TE buffer was added to the recovered pellet.

Gene Amplification for PCR-RFLP Analysis

Amplification of selected genes was adopted from primers used in previous PCR-RFLP studies on different species (Table 2).

Table 2: Primer used for gene amplification for PCR-RFLP.

Gene	Primer sequence (5' to 3') (F) Forward primer (R) Reverse primer	Expected size (bp)	Species and references
<i>rbcl</i>	TGT CAC CAA AAA CAG AGA CT (F) TTC CAT ACT TCA CAA GCA GC (R)	1400	<i>Pseudotsuga menziesii</i> , Hipkins <i>et al.</i> (1990)
<i>rpoB</i>	CTA AGG GGT TGT TGT GTA AC (F) AAT ATG CAA CGT CAA GCA GT (R)	1300	<i>Nicotiana tabacum</i> , Shinozaki <i>et al.</i> (1986)

Amplification Reaction

Amplification reactions were performed in a mixture of 25 μ L containing 1x buffer, 1.5 mM MgCl₂, 0.2 mM dNTP, 0.5 μ M primers, 1U *Taq* polymerase and approximately 100 ng DNA. Thermalcycler was programmed for 2 min initial denaturation (94 °C), 1 min denaturation (94 °C), 1 min for primer annealing (55.3 °C) and 2 min for elongation (72 °C). The cycle was repeated 35 times. The PCR products were analyzed by gel electrophoresis in 0.8% agarose gel and stained with ethidium bromide to observe the amplicon.

Purification of Polymerase Chain Reaction (PCR) Product

PCR products may contain primer dimers and the remnant of PCR reaction mixture such as dNTPs and *Taq* polymerase. For further analysis, the products need to be purified to wash away the contaminants. PCR products were transferred to 1.5 mL tube and 0.1 volume of sodium acetate (pH 5.5) and 2.5 volume of absolute ethanol were added subsequently. The mixture was cooled at -20 °C for 30 min or more, before it was centrifuged at 12000 rpm for 10 min. Pellet containing DNA was collected and rinsed with 70% cold ethanol. The DNA pellet was dried at 37 °C in an oven for 2 hours and solubilized in 50 μ L TE buffer.

Digestion by Restriction Enzymes

The purified PCR products were subjected to digestion with restriction enzymes by following the manufacturer's protocol (Promega®). For complete digestion, the mixture was incubated according to its recommended incubation temperature for 1 to 4 hours. Eight restriction enzymes *DraI*, *HindIII*, *AluI*, *HspII*, *RsaI*, *NaeI*, *TaqI* and *SmaI* were chosen at random. The digestion product was separated in a 1.2% agarose gel and stained with ethidium bromide (0.5 μ g/ μ L). Banding patterns were observed under UV transillumination.

RESULTS

Amplification of *rbcl* and *rpoB* target regions generated approximately monomorphic fragments of 1400 bp and 1300 bp, respectively. The *rbcl* and *rpoB* amplified fragments were successfully digested with *Hind*III and *Rsa*I enzymes, respectively. However, all PCR products obtained were found to not contain restriction sites for *Dra*I, *Alu*I, *Hsp*II, *Nae*I, *Taq*I and *Sma*I. Digestion of the amplified *rbcl* region with *Hind*III produced two restriction patterns at 886 bp and 452 bp. The *rpoB* region was positively digested with *Rsa*I into three different fragments; with the size of 620, 380 and 50 bp (Table 3).

Table 3: The presence of restriction site in *rbcl* and *rpoB*.

Restriction enzymes	<i>rbcl</i>	<i>rpoB</i>
<i>Hind</i> III	886 bp 452 bp	-
<i>Rsa</i> I	-	620 bp 380 bp 50 bp

DISCUSSION

The *Hind*III and *Rsa*I enzymes successfully produced a discrete banding pattern in two different *rbcl* and *rpoB* regions. However, in each of the chloroplast gene region, all of the six *F. deltoidea* varieties shared a similar banding pattern. This result suggests that there is no restriction site variation among the six varieties of *F. deltoidea*. As PCR-RFLP only considers fragment sizes, it may not represent that all *F. deltoidea* varieties shared the same sequences. It was proven when the sequence analysis that determines *F. deltoidea* var. *intermedia* has its own identical sequences, but the other five varieties shared the same bases. *F. deltoidea* var. *intermedia* showed its own identical sequences because five substitutions occurred in the *rbcl* region and one substitution occurred in the *rpoB* region.

The *rbcl* and *rpoB* regions appeared as highly conserved DNA regions due to the lack of variation that occurs in chloroplast DNA markers within *Ficus* species (Rønsted *et al.*, 2007). It may be due also to little variation that occurs in size, structure and gene content of chloroplast DNA in angiosperm species (Olmstead and Palmer, 1981). However, PCR-RFLP of cpDNA has been successfully applied in upper taxa levels such as in *Cornus* (Chase *et al.*, 1993) and Dipterocarpaceae (Tsumura *et al.*, 1996). Hillis *et al.* (1996) suggested that PCR primers that amplified effectively for one species are not necessarily suitable for other species. Other than that, PCR produces less than 2 kb and random selection of primers to digest it might not be useful, as the band patterns produced tend to be uniform. In addition, it is expected that varieties relationship is closer than species so the use of longer universal and more specific PCR products should be developed in the future. This study provides evidence that PCR-RFLP was not an efficient tool for the detection of genetic variations between varieties in *F. deltoidea*. The principal limitation of this technique arises from the choice of suitable primers. Further study was suggested by using inter generic region or inter spacer region that evolve more rapidly than cpDNA, to determine the monophyletic taxa of *F. deltoidea*.

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